Published in final edited form as:

Biochem Biophys Res Commun. 2004 October 29; 323(4): 1293–1298. doi:10.1016/j.bbrc.2004.09.006.

Immediate early gene X-1 interacts with proteins that modulate apoptosis

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Abstract

Immediate early gene X-1 (*IEX-1*) modulates apoptosis, cellular growth, mechanical strain-induced cardiac hypertrophy, and vascular intimal hyperplasia. To determine how *IEX-1* alters apoptosis, we performed yeast two-hybrid studies using *IEX-1* as the “bait” protein, and examined interactions between *IEX-1* and proteins expressed by a human kidney cDNA expression library. We found that *IEX-1* interacts with several proteins of which at least four are known to play a role in the regulation of apoptosis: (1) calcium-modulating cyclophilin ligand; (2) tumor necrosis factor-related apoptosis-inducing ligand (tumor necrosis factor superfamily, member 10); (3) ML-1 myeloid cell leukemia gene encoded protein; and (4) BAT3, a gene present in the major histocompatibility complex. Our data suggest that *IEX-1* may regulate apoptosis by directly interacting with various proteins involved in the control of apoptotic pathways.

Keywords

IEX-1; gly96; 1,25-Dihydroxyvitamin D; Apoptosis

The human immediate early gene X-1 (*IEX-1*, also known as *Dif2*, and murine orthologs *gly96* and *PRG1*) plays a role in the control of apoptosis and cellular growth [1-20]. The gene (*gly96*) was initially identified in mouse fibroblasts as a serum-regulated, glycosylated, immediate early gene [1]. The messenger RNA of a similar gene (*p22/PRG1*) is induced in rat pancreatic cells by pituitary adenylate cyclase activating peptide (PACAP) [20]. The human ortholog of *gly96* and *PRG1*, *IEX-1*, was identified as an X-radiation induced in fibroblasts by Kondratyev et al. [2], and as a UV-radiation and 1 α , 25-dihydroxyvitamin D₃-regulated gene in human keratinocytes [3,4].

Increased expression of *IEX-1* is associated with an increase in the growth rate of keratinocytes and HeLa cells, and disruption of *IEX-1* gene expression in HeLa cells and 293 cells is associated with a decrease in cellular proliferation [3,7,8,21]. *IEX-1* has been shown to be induced in cardiomyocytes and vascular smooth muscle cells by mechanical stretch, and overexpression of *IEX-1* in vascular smooth muscle cells protects against stretch-induced hypertrophy [9,15]. Injury-induced neo-intimal and vascular smooth muscle proliferation is reduced by transfection of carotid vessels with *IEX-1* [10].

The rate of apoptosis is either enhanced or diminished depending upon the cell line and apoptosis-inducing reagent used. For example, in keratinocytes [22], HeLa cells [7], hepatocyte (Hc) [19], and 293 cells [8], IEX-1 increases the rate of apoptosis. With respect to the induction of apoptosis, the effect of IEX-1 can be explained partially by its inhibitory effect on survival signals. There is inhibition of TNF- α -induced activation and expression of phosphatidylinositol 3-kinase (PI3K)/Akt and blockade of expression of ML-1 myeloid cell leukemia gene encoded protein (MCL1), an anti-apoptotic BCL-2 family member, which is located downstream of Akt [19]. In some situations, expression of the gene in lymphocytes and Jurkat cells [11,12] results in a reduction of apoptosis and protection against TNF-induced apoptosis. Transgenic animals over-expressing IEX-1 in T-cells develop an extended duration of an effector phase of a specific immune response, and a lupus-like syndrome and apoptosis in T-lymphocytes is reduced [13,23].

IEX-1 is regulated by a variety of factors. IEX-1 expression is increased by serum, X-, and UV-irradiation, growth factors such as epidermal growth factor, inflammatory stimuli such as lipopolysaccharide and ceramide, retinoids such as all-*trans*-retinoic acid or *cis*-retinoic acid, and by over-expression of Sp1 in cells [1-3,6,10,11,20,24-31]. The gene is repressed by steroid hormones such as 1 α , 25-dihydroxyvitamin D₃ and by over-expression of p53 [3,4,30,32]. 1 α , 25-Dihydroxyvitamin D₃ also results in a redistribution of IEX-1 from the nucleus into the peri-nuclear and cytoplasmic space. The ratio of Sp1 to p53 within the cell appears to regulate the amount of IEX-1 expression present within the cell [30]. Slight modifications of IEX-1 transcription are observed by mutating putative elements for p300, Sox, NF κ B, and AP4 within the promoter of the IEX-1 gene [30].

To gain further insights into the regulation of apoptosis by IEX-1, we performed yeast two-hybrid studies using IEX-1 as a “bait” protein, and a human kidney cDNA expression library as the source of expressed proteins. We now show that IEX-1 interacts with four proteins that are involved in the regulation of apoptosis, namely, calcium-modulating cyclophilin ligand (CAML), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, tumor necrosis factor super family, member 10), ML-1, a myeloid leukemia cell gene encoded protein, and BAT3. A fifth protein, zyxin, which is part of the focal adhesion plaque, has recently been shown to modulate apoptosis as well.

Materials and methods

General

pGBKT7 and pACT-2 yeast expression vectors and a human kidney cDNA library (S1769, Lot No. OO60199) were purchased from Clontech (Palo Alto, CA). Protein A/G-Sepharose beads were purchased from Pierce (Rockford, IL). Anti-CAML Mouse Monoclonal Antibodies were generous gifts from Dr. Richard Bram (Mayo Clinic, Rochester). Antibodies against human IEX-1 were generated as described earlier [33]. Protein concentrations were measured using the Bio-Rad reagent (Bio-Rad Laboratories, Hercules, CA).

Yeast two-hybrid screening

Yeast two-hybrid screening was performed as described by the manufacturer (Clontech Matchmaker Gal4 System 3, BD, Clontech, Palo Alto, CA). Full-length human *IEX-1* complementary DNA generated by polymerase chain reaction methods and encompassing the *IEX-1* coding region was ligated into *Eco*RI and *Bam*HI sites of the yeast two-hybrid GAL-4 DNA-binding domain (BD) expression vector, pGBKT7 [34]. The chimeric plasmid was used to express the “bait” protein IEX-1 in *Saccharomyces cerevisiae* AH109 cells. A human kidney cDNA library was prepared by the manufacturer using the GAL-4 activation domain (AD) pACT-2 vector (Clontech BD Bioscience, S1769, Lot No. OO60199) and expressed proteins

were examined for interactions with IEX-1. Preliminary experiments were carried out to show that IEX-1 expression does not activate transcription of genes itself in *S. cerevisiae* AH109 cells and that the IEX-1 protein is expressed in *S. cerevisiae* AH109 cells.

Saccharomyces cerevisiae

AH109 (MAT α) cells were transformed with the chimeric IEX-1- pGBKT7 DNA-BD plasmid encoding full-length human IEX-1, and transformed cells were mated for 24 h at 30 °C with *S. cerevisiae* Y187 (MAT α) cells pre-transformed with a human kidney cDNA library (S1769, Lot No. OO60199). The mating mixture was plated onto SD/-His/-Leu/-Trp selective medium plates. Plates were incubated for 8 days at 30 °C. For further selection of true positive clones, yeast colonies were re-plated onto SD/-Ade/-His/-Leu/-Trp plates containing X- α -Gal. The plates were incubated for 5 days at 30 °C. Positive blue colonies were picked and plasmid DNA was isolated and used to transfect *Escherichia coli* cells. Transformed *E. coli* cells were grown on an ampicillin containing nutrient agarose plate to select for clones containing the AD vector, pACT-2. Colonies were picked and the individual plasmids were amplified and purified [34]. Automated di-deoxy DNA sequencing [34,35] of purified plasmid DNA followed by analysis using the NCBI Blast Search program revealed the identity of insert DNA in positive clones.

Cell culture and stable transfection

HaCaT cells were routinely grown in high glucose Dulbecco's modified Eagle's media and supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO₂ atmosphere. These cells were stably transfected with full-length human IEX-1 cloned into the pcDNA 3.1 Zeo (-) vector as described previously [3]. Zeocin (400 μ g/ml) was used to select transformed cells.

Immunoprecipitation and immunoblotting

For immunoprecipitation, transfected HaCaT cells were washed with 1 $\times$ PBS and lysed in lysis buffer (300 mM NaCl, 50 mM Tris, pH 7.4, 1% Triton X-100, 5 mM EDTA, 0.02% sodium azide, 1.0 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). Cell lysates were pre-cleared for 30 min at 4 °C with protein A/G beads. Immunoprecipitation was performed using protein A/G beads conjugated to either anti-CAML or anti-IEX-1 antibodies by incubation for 2 h at 4 °C. Pre-cleared lysates were mixed with antibody labeled beads and incubated overnight at 4 °C. Beads were centrifuged briefly at 4 °C and washed three times with ice-cold washing buffer (0.1% (w/v) Triton X-100, 50 mM Tris, pH 7.4, 300 mM NaCl, and 5 mM EDTA). Interacting proteins were heated at 100 °C in 30 μ l of SDS-sample buffer, loaded onto 15% SDS-polyacrylamide gel, and analyzed by immunoblotting. The PVDF membrane was blocked in a 1% TBS blocking solution (Roche Molecular Biochemicals, Indianapolis, IN) for 1 h at room temperature with agitation. Membranes were blotted using anti-IEX-1 antibodies (1:1000) or anti-CAML (1:500) primary antibodies for 2 h at room temperature. Membranes were then washed and incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at room temperature. Proteins were detected using BM chemiluminescent detection solution (Roche Molecular Biochemicals).

Results and discussion

Sixty-three yeast colonies showed histidine, adenine, tryptophan, and leucine auxotrophy. Of these 63 colonies, 13 colonies grew and turned blue when grown on SD/-Trp/-Leu/-His/-Ade + X- α -Gal plates. The method used to verify interaction between proteins was stringent, in that initial colonies growing on media lacking in tryptophan, histidine, and leucine were further screened on media lacking tryptophan, histidine, leucine, and adenine. Finally, healthy colonies growing on the latter plates were further tested for their ability to hydrolyze X- α -Gal and

generate blue colonies. These colonies were evaluated further by sequencing of inserts. The identity of the DNA inserts was determined by NCBI blast searches.

To further verify the authenticity of the interactions seen in the two-hybrid system we performed immunoprecipitation reactions with IEX-1 and CAML specific antibodies, which were used to immunoprecipitate the proteins from human keratinocytes (HaCaT cells). When lysates of these cells were treated with anti-IEX-1 antibody beads, CAML was immunoprecipitated with IEX-1 and was detected with an anti-CAML antibody. It is very likely that the interactions that were seen in this system are indeed authentic and of biological relevance.

Sequencing of inserts in the pACT2 plasmids showed the following inserts that encoded proteins of interest: calcium-modulating cyclophilin ligand, TNF-related apoptosis-inducing ligand (TRAIL), the BCL2 homolog MCL1, BAT3, zyxin, platelet factor 4 variant, HUEL (c4orf1), peroxisomal enoyl coA hydratase, small glutamine rich tetratricopeptide containing protein, integrin $\beta 5$ precursor, and the β polypeptide of the sodium potassium ATPase. Remarkably, the first four proteins (out of a total of eleven) are involved in the regulation of apoptosis [36-63] (see Table 1 for a summary of the actions of these proteins). A fifth protein, zyxin, has also been implicated in the apoptotic response [64,65]. Since IEX-1 has been shown to modulate apoptosis, this is of great interest as it points to mechanisms by which IEX-1 may function in this regard.

Calcium-modulating cyclophilin ligand is an integral membrane protein that appears to be a participant in the calcium-signaling pathway [36-41,43]. CAML functions in a manner similar to cyclosporine A, binding to cyclophilin B and acting downstream of the T cell receptor and upstream of calcineurin by causing an influx of calcium. CAML is thought to inhibit the rate of apoptosis in some cells [43]. TNF-related apoptosis-inducing ligand [TRAIL/Apo-2 ligand, tumor necrosis factor, member 10 (TNF SF10)] is a member of the tumor necrosis family that induces apoptosis [44-49]. The BCL2 homolog, MCL1, is expressed in human myeloid leukemia cell lines during phorbol ester-induced differentiation along the monocyte/macrophage pathway. The protein has sequence similarity to BCL2. Expression of the *MCL1* gene is associated with inhibition of program cell death [50-53,55,56,58,60]. BAT3 is a 110 kDa protein that has an amino terminal ubiquitinlike domain, is rich in proline, and contains short tracks of polyproline, polyglycine, and charged amino acids. The gene for *BAT3* is present within the major histocompatibility complex and is closely linked to TNF- α , TNF- β , and HLA-B genes. Zyxin, which is a component of adhesion plaques, has been suggested to perform regulatory functions at these specialized regions of the plasma membrane. It displays a collection of proline rich sequences as well as three copies of the LIM domain, a zinc finger domain found in many signaling molecules. Recent findings suggest that zyxin may play a role in CD99 associated signaling in Ewing's sarcoma cells. Treatment of ES cells with zyxin antisense oligonucleotides inhibited CD99-induced cell aggregation and apoptosis, suggesting a role for zyxin in CD99 function [64]. Additional data suggest that zyxin like domains may be involved in VASP and semaphorin cell signaling [65].

IEX-1 enhances apoptosis in some cells, whereas in other cell systems, IEX-1 protects against apoptosis [7,8,11-13,19,22,23]. Our findings show that IEX-1 may function in the control of apoptosis by directly binding to a variety of proteins associated with either the enhancement or inhibition of apoptosis and that differential binding and activation may provide an explanation for the varied effects of this protein in different cells. Further investigations of the mechanism of action of IEX-1 involving binding to pro- or anti-apoptotic molecules are warranted.

Acknowledgments

This work was supported by NIH Grant DK25409.

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Table 1

Identity DNA inserts in IEX-1 interacting clones and function of encoded proteins

Clone No.	Identity of DNA	Accession No.	Function of encoded protein—comments from NCBI summaries in nucleotide database
1	<i>Homo sapiens</i> calcium modulating ligand	NM_001745	The integral membrane protein encoded by this gene functions similar to cyclosporin A, binding to cyclophilin B and acting downstream of the TCR and upstream of calcineurin by causing an influx of calcium
2	TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand); <i>Homo sapiens</i> tumor necrosis factor (ligand) superfamily, member 10, (TNFSF10)	NM_00381	The protein encoded by this gene is a cytokine that belongs to the tumor necrosis factor (TNF) ligand family. TRAIL induces apoptosis in transformed and tumor cells, but does not normal cells. TRAIL binds to several members of TNF receptor superfamily
3	<i>Homo sapiens</i> myeloid cell leukemia sequence 1 (BCL2-related) (MCL1)	NM_182763	The protein encoded by this gene belongs to the Bcl-2 family. Alternative splicing occurs at this locus and two transcript variants encoding distinct isoforms have been identified. The longer gene product (isoform 1) enhances cell survival by inhibiting apoptosis while the alternatively spliced, shorter gene product (isoform 2) promotes apoptosis and is death-inducing
4	<i>Homo sapiens</i> HLA-B associated transcript 3 (BAT3)	NM_080703	The BAT1-BAT5 gene cluster is present in the vicinity of the genes for TNF- α and TNF- β . These genes are all within the human major histocompatibility complex class III region. The protein encoded by this gene is a nuclear protein. It has been implicated in the control of apoptosis and regulating heat shock protein. There are three alternatively spliced transcript variants described for this gene